GENERAL PROTOCOL FOR RESIDUE ANALYSIS OF AVERMECTIN B1 AND ITS DELTA 8,9 ISOMER

AB-P1
REVISION 1

MARCH 15, 1989

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I. INTRODUCTION

Avermectin B1 (abamectin) and its delta 8,9 isomer are determined in a specified sample matrix using the appropriate Merck Sharp and Dohme Research Laboratories (MSDRL) method for that sample matrix. Procedural details for determining abamectin residues are described in the method. The intent of this general protocol is to summarize the criteria by which residue data should be generated and evaluated. This protocol serves as a guideline to what is considered to be good laboratory practices for residue analysis at and for MSDRL. This does not substitute for good scientific practice or judgement and in no way should be used to generate anything but the best residue data possible. Adhering to this protocol does not guarantee acceptability of the data at Merck or any regulatory agency, but such adherence should minimize misunderstandings.

Avermectin B1 consists of a mixture of two homologs, containing not less than 80% avermectin B1a and not greater than 20% avermectin B1b. These components differ only by one methylene unit ($-CH_2-$) at the 25 carbon position; B1a contains a sec-butyl group and B1b contains an isopropyl group. Characterization of the total toxic residue in a variety of agricultural matrices has determined that a small part of the residue also consists of the photodegradate avermectin B1 delta 8,9 (Z) isomer. Consequently, the analytical methods have been devised to also quantitate the delta 8,9 isomer. The methods use high pressure liquid chromatography (HPLC) to quantitate two fluorescent derivative peaks; one which is formed from avermectin B1a plus from its delta 8,9 isomer and one which is formed from avermectin B1b and its analogous isomer. Further details about the method chemistry are found in the individual method descriptions.

The MSDRL method details the materials and procedures to be used. Solvent, reagent, glassware, etc. suppliers may be changed depending on the availability and practicality. Only materials and equipment which have been adequately tested may be substituted. Certain equipment and reagents may be substituted only with the approval of the MSDRL Analytical Research Department; these substitution guidelines are listed in the appropriate method.

II. METHOD VALIDATION

A. Full Validation

All abamectin residue methods will be fully validated, documented and approved before sample analyses are begun. Usually the "full" validation will be completed within Analytical Research prior to transmitting the method to a contract laboratory for routine assays. The "full" validation will consist of a series of quintuplicate samples run through the entire method. Generally, this series will consist of:

a. 5 control samples (untreated sample matrix)
b. 5 fortified control samples at the quantitation limit for B1a specified in the method
c. 5 B1a fortified control samples at approximately five times the quantitation limit
d. 5 B1a fortified control samples at approximately ten to twelve times the quantitation limit
e. 5 B1b fortified control samples at the quantitation limit (usually obtained concurrently from d.)
f. 5 delta 8,9 isomer fortified control samples at the quantitation limit
g. 5 delta 8,9 isomer fortified control samples at approximately five times the quantitation limit
h. 5 delta 8,9 isomer fortified control samples at approximately ten to twelve times the quantitation limit

At all times the fortification level will be at or above the quantitation limit. In most crop matrices, the quantitation limit is 5 ng/g which means the full validation series is:

a. 5 controls
b. 5 fortified at 5 ng/g B1a
c. 5 fortified at 25 ng/g B1a
d. 5 fortified at approximately 70 ng/g B1a
e. 5 fortified at approximately 5 ng/g B1b
f. 5 fortified at 5 ng/g delta 8,9
g. 5 fortified at 25 ng/g delta 8,9
h. 5 fortified at approximately 70 ng/g delta 8,9.

B. Limited Validation

A more limited validation is required of the contract laboratory and individual analysts before sample analyses are begun. This limited validation series of samples will include:

i. 3 control samples (from untreated matrix)
ii. 3 B1a fortified control samples at the quantitation limit.
iii. 3 B1a fortified samples at approximately 10-12 times the quantitation limit
iv. 3 B1b fortified samples at the quantitation limit
v. 3 delta 8,9 isomer fortified samples at the quantitation limit
vi. 3 delta 8,9 isomer fortified samples at five times the quantitation limit

The results of this limited laboratory validation will be conveyed to MSDRL Analytical Research in a complete report (described below), subject to audit by Merck. Additional validation samples are recommended, if the above data are borderline or if the laboratory is not totally comfortable with the method.

An analyst new to the method should also complete the limited validation before sample analyses are begun. This supplemental validation, including all raw data, must be kept on file and available for review. The results should be summarized in a letter or memo to the Merck sponsor to document that the analyst is validated. It is strongly recommended that any analyst who has not performed the method in more than six months conduct a similar limited validation, to verify that the method still works in his or her hands.

C. Validation Criteria

The method validation (limited or full) is subject to all of the restrictions described below for routine sample analysis, including the performance characteristics (e.g. method recovery) and the set size. No deviations from these requirements are permitted without the written consent of the study monitor in MSDRL Analytical Research. Any set of data which do not meet the requirements shall be rejected and repeated.

Method recoveries for the avermectin B1a, delta 8,9, and B1b fortifications must be between 70-110%. For any given sample set during the validation, only one fortified sample out of six may fall outside the acceptable recovery range. If this ratio (1/6) is exceeded, the entire sample set must be repeated. The sample which is out of range is unacceptable and must be repeated in all cases.

The untreated control matrix to be used for validation normally should be from authentic field samples, representative of the quality and maturity of trial samples, Control matrix should not be obtained from a grocery store, unless no untreated field samples are available and unless such use is approved by MSDRL Analytical Research.

The successful method validation must be completed, reported and approved by MSDRL Analytical Research before routine sample analyses are begun.

III. RECEIPT AND STORAGE OF SAMPLES

A. MSDRL (Mr. A. Macaoay, 201-369-3072 or alternate designated on shipping papers) must be notified by phone or facsimile transmission (FAX number 201-369-8811) as soon as samples arrive. All samples will be logged in no later than 48 hours after receipt with all information given on the sample bag or container, matched to a unique laboratory code (which could be the protocol number). Exceptions to the timing for login (such as for delays due to weekends or holidays) may be made with prior approval of MSDRL Analytical Research.

B. Immediately following login, written confirmation of sample receipt should be forwarded (to Mr. Macaoay or alternate), including the date of arrival, the sample list including all assigned laboratory codes and a description of the samples' condition upon arrival. Generally, a form will be provided before or with the sample shipment to be completed with this information and returned (to Mr. Macaoay or alternate). If there are any problems with the samples or the shipment, MDSRL (Mr. Macaoay or project coordinator) should be telephoned immediately.

C. Unless otherwise specified, samples are to be stored frozen at or below -10 degC between receipt, processing and analysis. Documentation of freezer temperature must be retained and available upon request. Deviations from these storage requirements must be adequately explained.

D. Samples will be logged out each time they are removed from the storage freezer. The person responsible for the sample and the date logged out will be recorded as well as the reason for removal. Documentation of the sample storage log must be retained and available upon request.

IV. SAMPLE PREPARATION

A. The procedures used to prepare the sample differ depending on the sample matrix. Deviations to this general sample preparation regimen are detailed in either the analytical method for the matrix or in a separate protocol for the specified matrix. Variations may be requested in writing by MSDRL Analytical Research. Compositing of samples are described in separate protocols appropriate to the matrix.

B. Solid samples are usually shipped frozen in plastic, impregnated cloth bags. Liquid samples are usually shipped frozen in polyethylene bottles. The sample size depends on the matrix and is generally specified in the study protocol.

C. The processing procedure is designed to combine individual fruit, plants, nuts, cores, etc. into one homogeneous representative sample which is amenable to further analysis. Samples should be processed frozen, if feasible, but may be partially thawed, if necessary for grinding or processing. Appropriate measures should be taken to ensure no loss of residue from the samples and to maintain sample label integrity (see section F below) on the original or subsequent containers.

D. Samples should be processed in an order to assure no sample cross contamination. Generally, this means control samples should be processed first, followed by the later interval and/or lower treatment level samples. The last samples processed usually should be the samples with the highest expected residue values (usually Day 0). The order of processing may be adjusted at the request of MSDRL Analytical Research.

E. The samples are ground to a homogeneous blend using a Cuisinart food processor, Hobart food chopper or equivalent apparatus (approved by MSDRL Analytical Research) designed to thoroughly mix samples. The apparatus must be adequately cleaned between samples to minimize possible sample contamination. The procedure for cleaning the apparatus should be documented (such as in an SOP) and available for review.

F. The date of processing for each sample is to be recorded for the study report. Approximately 500 grams of the sample homogenate should be retained in a labelled 500 ml wide mouth square polyethylene (e.g. Nalgene) bottle with a screw cap. Other approved containers may be substituted for the Nalgene bottles. Approximately 1000 grams of control samples should be retained in square polyethylene bottles. Archive samples are prepared from the sample homogenates by retaining approximately 100 grams representative of the samples in appropriately-sized square polyethylene bottles labelled with all necessary information, including the designation in large letters "ARCHIVE". (If the samples are smaller than specified above, the maximum amount possible should be retained.) The information to be included on the bottle labels includes all details noted on the sample bag plus any additional information necessary to uniquely identify the sample. The MSDRL sample bags are currently designed with tear-off labels, which may be used to ease the data transfer. If there are any questions about sample identity or condition, the sample bags should be retained to assist the investigation resolving these questions. The integrity of sample identification labels on the containers should be maintained by the use of indelible marking pens and clear tape to cover and preserve the label during handling.

G. The waste or sample remaining after homogenization (if any) and after transfer into the storage or archive containers should be discarded appropriately unless otherwise specified, if the sample is from an unrestricted US study. All samples and waste from international studies must be returned to MSDRL-Three Bridges for disposal appropriate under the import permit, unless the processing lab received other instructions. Samples or waste from US restricted studies usually will be returned to MSDRL-Three Bridges for disposal, except when other instructions are provided. (See section I. below about returned items.) Information about whether a study is imported or restricted will be provided by Merck before the samples arrive or with the sample shipment. NOTE: Disposal of any samples and waste must comply with all appropriate local, state and Federal regulations. All boxes, bags, etc. which have the Merck name on them should have the name obliterated before disposal (unless the items are incinerated). This policy is designed to avoid the inappropriate reuse of these items.

H. Samples are to be stored at or below -10 degC at all times except during analysis (see section III-C.). Before subsampling for analysis, the sample should be thawed or partially thawed and mixed thoroughly, as appropriate for the matrix, to ensure that a representative sample is aliquoted.

I. Upon completion of the study (including approval of the final report), the archive samples are to be returned to MSDRL-Three Bridges. Upon notification of MSDRL receipt of the archive samples, the remaining samples may be appropriately discarded or returned to MSDRL, as specified by Analytical Research, MSDRL. Disposition of samples and all concomitant waste will be documented by indicating the date and means of disposal or shipment. This documentation should be sent within seven days to Analytical Research, MSDRL to complete the sample history. All samples and waste should be returned or disposed of immediately upon Analytical Research, MSDRL request. No samples or waste should be shipped to Analytical Research, MSDRL without prior notice of the exact date and means of shipment. It is recommended that approval be obtained from Analytical Research before arrangements are made for shipment.

V. ORDER OF ANALYSIS FOR FIELD SAMPLES

A. Unless otherwise specified, processed field samples are assayed from consecutive sampling dates, starting with the first sampling interval after the 0 (or 2 hour) day (e.g. day +1).

B. For each treatment group, sample analysis continues until two consecutive sampling intervals contain no detectable residues in all replicates or in composited samples.

C. After the conditions in B. are satisfied, the day -1 (if any) and day 0 samples, in that order, are assayed, with due care to avoid cross contamination.

D. The order of sample analyses may be adjusted at the request of MSDRL-Analytical Research and only should be adjusted with the permission of MSDRL-Analytical Research.

VI. QUANTITATION OF AVERMECTIN B1 AND ITS DELTA 8,9 ISOMER

Avermectin B1 residues are quantitated by comparison with a standard curve of avermectin B1a. Research at MSDRL has demonstrated that derivatization of avermectin B1a delta 8,9 isomer results in the same derivative as for avermectin B1a. Thus, the derivatized residue at the retention time of B1a represents the sum of residue for avermectin B1a and its delta 8,9 isomer. Additional research has shown that essentially equivalent results for avermectin B1b are obtained using either a B1a or a B1b standard curve. Because B1b is at most 20% of the active ingredient, its residue levels are normally quite low and are always lower than the avermectin B1a + delta 8,9 residue levels.

The exact retention time for the derivatives will vary with the chromatographic apparatus, but in general, if the avermectin B1a derivative elutes at approximately 10 minutes, the avermectin B1b derivative elutes at approximately 8.5 minutes. The specific retention

time for B1b is determined from the B1b peak observed in the highest B1a standard.

The results for the two different derivatives should be reported separately, as specified in the method or study protocol, but the quantitation procedure is the same for both derivatives. A linear regression fit is determined for the standard curve (with the peak height as the ordinate and the concentration as the abscissa), calculating the slope and intercept for the avermectin B1a curve. The specific formula to be used in the calculation of residue levels is listed in the appropriate method, but generally takes the form of:

$$C = (PK\ HT - I) / S$$

$$UNK = (C \times FV) / (SW \times FRAC)$$

where
C = concentration of avermectin B1 derivative in ng/ml in the final volume used for the HPLC analysis.
PK HT = peak height of derivative.
I = intercept and S = Slope of standard curve.
FV = final volume used in the HPLC analysis.
UNK = concentration of avermectin B1 (usually ng/g) in the unknown sample.
SW = sample amount, usually weight in grams
FRAC = fraction of sample used in the HPLC assay

VII. ANALYSIS OF LABORATORY SAMPLES

A. Analysis Sets

An analysis set consists of no more than 12-15 samples, the exact number depending on the method and the experience of the analyst. Each analysis set will contain, from the beginning of the assay procedure, at least one control (untreated) sample and at least one control sample fortified to determine method recovery in the set. Only one analyst should be involved in the manipulations of the analysis set.

Unless otherwise permitted by MSDRL, the control samples to be used should be from the study being assayed. An injection set consists of an analysis set of samples injected with five standards which were derivatized at the same time as the samples. The standards are usually injected as a group before and after the samples, resulting in a total of 22-25 injections in the set. Unless otherwise specified by the method or MSDRL, the analysis set size should not be increased. The injection set size can only be increased when sample dilutions are known to be necessary. In that case, the number of samples injected between standards should be at maximum 12-15 and an additional group or groups of the standards will need to be injected, to "bracket" the samples. However, it is usually preferable to treat the injections of different dilutions from the same samples as a separate injection set(s).

In some instances, such as for robotic assays, it may be appropriate to intermingle the standards with the samples. In these cases, the ratio for the number of standard injections to the number of sample injections should be maintained or exceeded.

B. Standards Calculations

Avermectin B1 standards will be prepared and derivatized according to the appropriate MSDRL method. Both (or all) groups of standards in an injection set will be used in the calculation to determine the linear regression coefficients (slope and intercept) and the coefficient of determination (r^2).

Occasionally it has been observed that both peak heights for one standard are much lower than expected. Because it is known that this observation can be attributed to a low derivatization reaction yield, both peak heights of the standard may be discarded in determining the regression coefficients and coefficient of determination. With appropriately detailed documentation of a scientifically sound explanation, all injections or a single injection of a standard may be discarded. At no time may more than one standard be discarded in an injection set. A standard should not be rejected unless there is an adequate explanation. The set should be reinjected if necessary to meet these criteria.

A coefficient of determination (r^2) greater than 0.97, based on a minimum of 8 data points (four different standards injected before and after the samples) is required. A coefficient of determination of less than 0.97 usually means that the derivatization should be repeated or that the analysis set should be repeated. Data points from a complete standard curve which meets these criteria should not be discarded just to improve the coefficient.

C. Recovery Samples

Fortified control samples should be prepared at an avermectin B1 concentration as close as is practical to the expected analyte concentrations of the study samples. If no residues are expected, the control sample should be fortified at the limit of reliable quantitation attributed to the method, usually 5 ng/g. Unless otherwise specified, fortified samples are prepared by adding an aliquot of standard (in acetonitrile) to the sample homogenate matrix before extraction, at the beginning of the assay procedure.

Avermectin B1 recovery from a fortified sample is required to be 70-110%. A recovery value of less than 70% or greater than 110% will require that the analysis set be repeated.

Residue data will not be corrected for recovery.

D. Quantitation of Unknown Samples

Quantified peaks must have a signal to noise ratio of greater than 5 and they must be within the peak height range of the standard curve. Peak

heights that are below the height of the lowest standard used are not quantifiable and peak heights that are above the height of the highest standard used are from samples which must be diluted and reinjected, to fall within the standard curve range and to be quantified. Good chromatography, as indicated by lack of interfering peaks and a steady baseline, is required for quantifying peaks.

For samples with measurable peak heights, but below the lowest standard, a calculation of the residue concentration is performed, to verify that the value is below the detection limit specified in the method (usually 2 ng/g). Samples below the detection limit are reported as "not detected" or ND. Sets containing samples which calculate to values above the detection limit but are below the lowest useable standard should be examined to determine if the set should be reassayed or reinjected. The lowest useable standard should adequately define the detection limit for the set and there should be no samples below it that contain detectable residue.

For samples with peak heights above the lowest standard, the avermectin B1 (B1a +delta 8,9 or B1b) residue should be calculated. Usually, B1 residues above 2 ng/g but below 5 ng/g will be reported as "not quantitated" (NQ). The calculated value must be recorded in the raw data table. The quantitation procedure and the definition of NQ (as well as that of ND) may vary depending on the method specifications.

As stated above, samples with peak heights above the highest standard's peak height must be diluted and reinjected with the same standards prepared with the set. Samples with peak heights below the lowest standard because of overdilution must be rediluted and reinjected.

E. Reinjection Sets

A fortified control (method recovery) sample from a set must be reinjected if any of the corresponding unknown samples are reinjected in another set. The reinjected value is used to demonstrate sample stability. The criteria for an acceptable set apply to reinjected sets, as well. The results from recovery samples injected more than once are to be averaged together in determining the overall recovery during the study. (This avoids biasing the overall recovery value by a particular sample which may have been injected more than the others.)

The untreated control sample of a set must be reinjected if the entire set is being reinjected or if it is the only acceptable sample in a set. If only part of the set needs to be reinjected (i.e. for dilutions), the control sample does not need to be reinjected unless there is reason to be concerned about contamination or poor chromatographic performance.

VIII. PREPARATION OF THE REPORT

The report should include the following information:

A. Project personnel
   1. Supervisor
   2. Analyst(s)
   3. Technicians and any personnel involved with sample storage, preparation, analysis or reporting.
   4. Quality assurance officer
   5. MSDRL's project supervisor

B. Quality assurance statement, as well as any critical phase monitoring

C. Project description, including assigned laboratory project number and Merck trial number. (The cover page should at least indicate the matrix, the Merck trial number and the report date.)

D. Description of sample receipt, storage conditions, etc.

E. Test method and deviations from the method, with reasons.

F. Identification of all standards and dilutions used for quantitation and fortification, by L-number, including appropriate descriptions of standards' preparation.

G. Summary tables of data should include, at minimum, when the information is available:

   1. Raw agricultural commodity
   2. Abamectin application rate and sampling interval
   3. Laboratory identification, if any
   4. Merck sample identification
   5. Dates sample processed, extracted and analyzed
   6. Analysis set number
   7. Amount detected expressed as ng avermectin B1a + delta B,9 or avermectin B1b per gram (usually) of sample. Values less than 5 ng/g but greater than 2 ng/g are reported as NQ. Values less than 2 ng/g are reported as ND. The method may specify other units or cutoff limits to be used, as appropriate for the matrix.
   8. Fortification levels
   9. Percent recovery for fortified samples

H. Raw data reported or tabulated for each injection set should include the following:

   1. Injection set and/or analysis set number
   2. Graphical representation of the standard curve, with ng/ml standard plotted as the abscissa and peak height plotted as the ordinate.

3. Regression coefficients for the linear least squares fit of the standards: slope, intercept and coefficient of determination.
4. Peak heights of all samples and standards
5. Laboratory identification of all samples analyzed.
6. Fortification levels in ng/g.
7. Microliters injected, sample final volume, including dilutions, and sample weight.
8. Dates for each sample processed, extracted and analyzed.
9. Amount detected (expressed as ng avermectin B1a/delta 8,9 or avermectin B1b per gram of sample, as appropriate for the method). Values containing no detectable residue will be reported as ND.
10. Individual chromatograms labelled with:
    a. analysis set
    b. laboratory sample identification
    c. date of analysis
    d. peaks of interest identified
    e. dilutions, if appropriate
    f. retention time of B1a/delta 8,9 and B1b (indicated on all chromatograms.)
    g. analyst identification (usually initials suffice)
    g. whatever else is necessary to uniquely identify the chromatogram and to track the sample
11. Description of instrumentation and operating conditions with sufficient details to allow the system and exact sample handling to be duplicated.

I. Reporting of results should reflect the appropriate number of significant figures. The list below contains examples of significant figures. Unless standard operating procedures at the analyzing laboratory dictate otherwise, one additional significant figure may be used in performing the calculations up to the final raw data result (residue concentration or r^2).
   1. One significant figure in the non-quantifiable concentration range, usually 2-5 ng/g; calculated as 1 sig. fig. but reported as NQ.
   2. Two significant figures in the region above NQ on the standard curve, such as for 5.0 - 9.9 ng/g.
   3. Three significant figures for concentrations at or above 10.0 ng/g.
   4. Recoveries below 100% should be reported with two significant figures; values above 100% should be reported with three significant figures.
   5. The procedure used for rounding, if different from these guidelines, should be described in the data section of the report.

J. All pages of the final report, including raw data, must be completely legible with margins of 1 - 1.5 inches on each edge. Reports which do not meet these criteria will be rejected.

METHOD NO. 8920

1924

618-936-93127

HPLC-FLUORESCENCE DETERMINATION FOR AVERMECTIN B_1 AND ITS 8,9 ISOMER IN CUCUMBERS

Method No. 8920

Merck Sharp and Dohme Research Laboratories
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October 25, 1989

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Method No. 8920 October 25,1989

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Method No. 8920 October 25,1989

HPLC-Fluorescence Determination For Avermectin B1 and its 8,9 Isomer in Cucumbers

Merck Sharp and Dohme Research Laboratories
Division of Merck & Co., Inc.
P.O. Box 2000, Rahway, N.J. 07065-0914
Method No. 8920
October 25, 1989

I. SUMMARY

Avermectin B1 (Abamectin) is a mixture of two homologs containing not less than 80% avermectin B1a and not greater than 20% avermectin B1b. These components differ by only one methylene unit (-CH2-) at the 25-carbon position, wherein avermectin B1a contains a sec-butyl group and avermectin B1b contains an isopropyl group.

The first analytical methods developed were for the companion animal health drug ivermectin (22,23-dihydro avermectin B1). As it was known that the avermectins were not amenable to gas chromatography and the available liquid chromatography methods employed UV detection which lacked the required sensitivity and selectivity, an HPLC method employing fluorescence detection was developed by Tolan et al. (1980) and modified by Tway et al. (1981) for ivermectin in tissue. The Tway method is directly applicable to avermectin B1. Following more complete characterization of the total toxic residue in a variety of agricultural matrices, it was determined that an analytical method would also be required for the photodegradate 8,9 isomer of avermectin B1. To assay for both the parent avermectin B1 and its 8,9 isomer, the Tway fluorescence derivatization was modified. Incubation at 30°C for 1 hour with the acylating reagent, trifluoroacetic anhydride, yields the fluorescent derivative shown in Figure 1 with the 4" position trifluoroacetylated (not shown). As this linkage is unstable, the trifluoroacetyl group at the 4" position is cleaved with methanolic ammonium hydroxide to give the stable 4"-OH fluoroderivative. This two step reaction yields the same fluorescent derivative for the 8,9 isomer as for the parent avermectin B1. Therefore, using this derivatization, the residue quantitated represents the sum of avermectin B1 and its 8,9 isomer.

Samples are prepared by extraction from the matrix with methanol and purified by liquid-liquid partition and solid

phase extraction. The resultant residue is derivatized and analyzed on an HPLC with fluorescence detection. Residues of avermectin B1/8,9 isomer below 2 ng/g are non-detectable (reported as ND). Residues between 2-5 ng/g are identified but not quantitated (reported as NQ) and residues above 5 ng/g are identified and quantitated from an avermectin B1a standard curve. Since avermectin B1b is at most 20% (normally less than 10 %) of the active ingredient, residues are generally present at levels less than the limit of quantitation (5.0 ng/g). At levels above 5 ng/g, avermectin B1b residues are quantitated in the same manner as avermectin B1a.

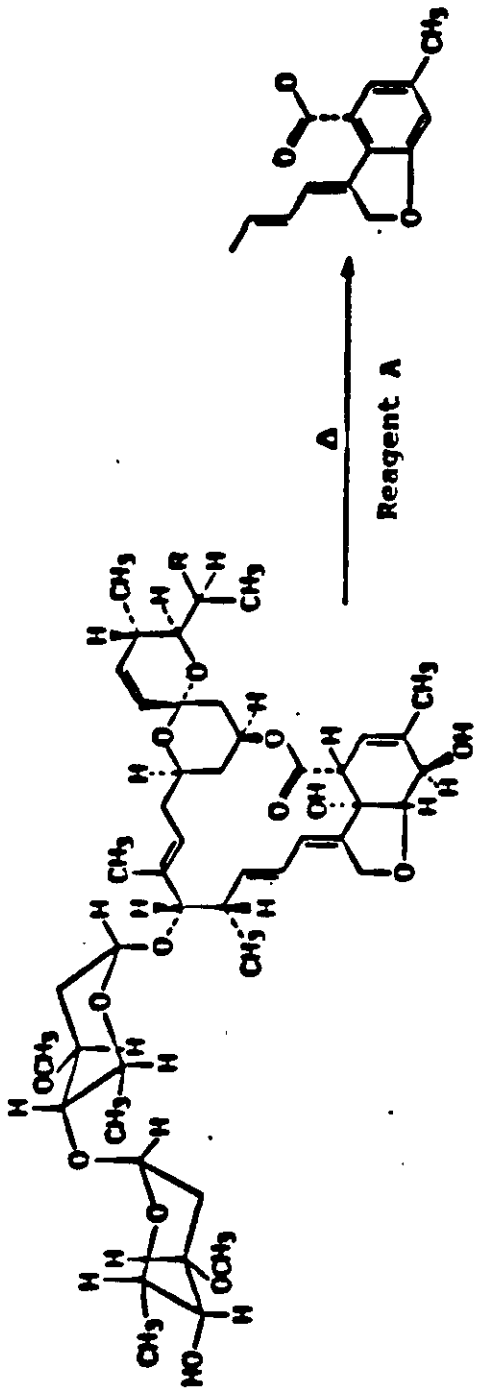


Figure 1. Structures of avermectin B1 and derivatization reaction

618-936-93127

II. METHOD VALIDATION

Table 1 gives the results of the validation of Merck Method No.8920. Homogenates of field grown control and store bought cucumbers were fortified at approximately 5, 25, and 90 ng/g with avermectin B1a and its 8,9 isomer, and at 6.6 ng/g with avermectin B1b. Several replicates (a minimum of 5) were prepared for each compound at each fortification level by adding 1 ml of the 50 ng/ml, 250 ng/ml, or 900 ng/ml working standards to 10 grams of the whole fruit homogenate just prior to the methanol extraction. As avermectin B1b is present in the avermectin B1 standard at approximately 10% of the avermectin B1a, 1 ml of the 900 ng/ml avermectin B1 standard (containing 900 ng/ml avermectin B1a and approximately 66 ng/ml avermectin B1b) was used for the avermectin B1b fortifications. Controls were fortified with 1 ml of the acetonitrile used to prepare the standards. For avermectin B1a (L-676,863-03BA002), recoveries averaged 92.6% (n=10, range 71-109%, standard deviation [S.D] 13.0%) for samples fortified at 5.4 or 5.9 ng/g, 100.0% (n=4, range 79-106%, S.D. 8.1%) for samples fortified at 25.5 ng/g, and 89% (n=5, range 86-94%, S.D. 3.6%) for samples fortified at 89.1 ng/g.

Avermectin B1b recoveries averaged 97% (n=5, range 92-105%, S.D. 5.7%) for samples fortified at 6.6 ng/g. [See Table 1.]

Recoveries for the 8,9 isomer were determined by spiking samples with the 8,9 isomer standard (L-652,280-002T001), derivatizing and quantitating versus the avermectin B1a derivatized standards. Recoveries of the 8,9 isomer from fortified control cucumbers at the 5.2 or 5.6 ng/g level averaged 75.7% (n=8, range 71-88%, S.D. 5.7%), at the 26.1 ng/g level recoveries averaged 70.8% (n=5, range 68-75%, S.D. 3.1%), and at the 52.2 ng/g level recoveries averaged 70.3% (n=5, range 67-72%, S.D. 2.2%). See Table 1 for detailed data on recoveries.

The average recovery for avermectin B1a for the concentration range from 5-89 ng/g avermectin B1a in cucumbers was 92.3% with an S.D. = 10.7%, n=19. The average recovery for the avermectin B1a 8,9 isomer for the concentration range from 5 to 52 ng/g in cucumbers was 72.5% with an S.D. =4.8%, n=18.

Shown in Figures 2 and 3 are example chromatograms for a cucumber control, a cucumber control sample fortified with avermectin B1a at 5.9 ng/g, an avermectin B1a 9.5 ng/ml

standard, and a cucumber control sample fortified with the 8,9 isomer of avermectin B1a at 5.2 ng/g.

Cucumbers acquired from a field in California and store bought cucumbers were used in the method development. These cucumber samples were representative of the most common cultivars of cucumber, and cucumber in different stages of maturity and ripeness. The recovery of the avermectins and chromatograms of the samples analyzed by the method were the same for all sample types. No interferences were found in any of the control or treated samples.

Table 1

Recovery of Avermectin B1a, Avermectin B1b, and the 8,9 Isomer from Cucumber Homogenates

SAMPLE NO	FORTIFICATION LEVEL ng/g	AMT FOUND ng/g	CALC % REC	
B1a				
65	0	0	NA	
77	0	0	NA	
83	0	0	NA	
89	0	0	NA	
101	0	0	NA	
113	0	0	NA	
66	5.9	5.7	97	
67	5.9	2.9	49*	
68	5.9	5.4	92	
69	5.9	5.9	100	
70	5.9	5.9	100	
107	5.4	5.9	109	
108	5.4	5.8	107	
114	5.4	4.1	76	
115	5.4	3.5	65*	
116	5.4	4.9	91	
117	5.4	3.8	71	
118	5.4	4.7	87	
			MEAN	92.6
			S.D.	13.0
95	25.5	20.2	79	
109	25.5	26.8	106	
110	25.5	26.8	104	
111	25.5	23.2	91	
			MEAN	100.0
			S.D	8.1
84	89.1	82.0	92	
85	89.1	83.4	94	
86	89.1	79.6	89	
87	89.1	77.7	87	
88	89.1	76.3	86	
			Mean	89.0
			S.D	3.6

Table 1 (con't)

Recovery of Avermectin B1a, Avermectin B1b, and the 8,9 Isomer from Cucumber Homogenates

SAMPLE NO	FORTIFICATION LEVEL ng/g	AMT ng/g	CALC % REC	
8,9				
78	5.2	3.9	75	
79	5.2	3.7	71	
80	5.2	3.9	75	
81	5.2	3.8	73	
82	5.2	3.8	73	
106	5.2	4.6	88	
112	5.2	3.8	73	
119	5.6	4.2	77	
120	5.6	3.9	70*	
			MEAN	75.7
			S.D.	5.7
90	26.1	18.4	70	
91	26.1	17.8	69	
92	26.1	18.6	71	
93	26.1	19.5	75	
94	26.1	17.7	68	
			MEAN	70.8
			S.D.	3.1
96	52.2	36.0	69	
97	52.2	35.1	67	
98	52.2	37.7	72	
99	52.2	37.1	71	
100	52.2	37.1	71	
			MEAN	70.3
			S.D.	2.2

Table 1 (con't)

Recovery of Avermectin B1a, Avermectin B1b, and the 8,9 Isomer from Cucumber Homogenates

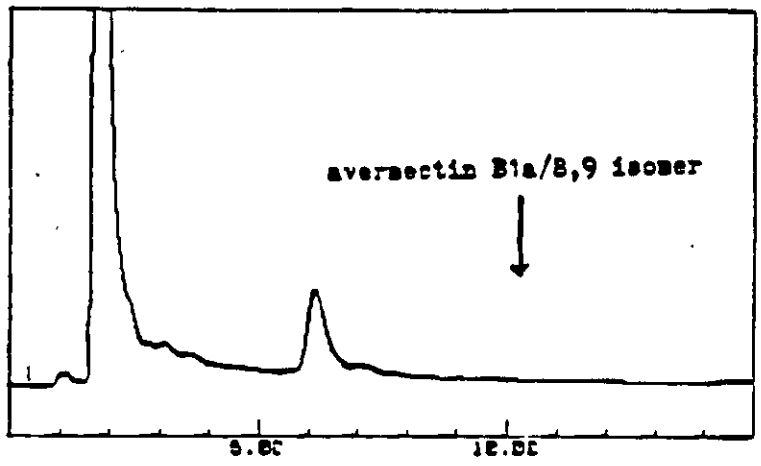
SAMPLE NO No.	FORTIFICATION LEVEL ng/g	AMT ng/g	CALC % REC
B1b			
84	6.6	6.7	102
85	6.6	6.9	105
86	6.6	6.4	97
87	6.6	6.1	92
88	6.6	6.2	94
			MEAN 97.0
			S.D. 5.7

Note * Sample did not derivatize completely. Results not included in the average.

Note: All control samples were free from any detectable avermectin residues.

Figure 2. Typical Chromatograms of Control Cucumber and Avermectin B1 in Cucumber.

CUCUMBER CONTROL SAMPLE 65



5.9NG/G B1a FORTIFIED CUCUMBER

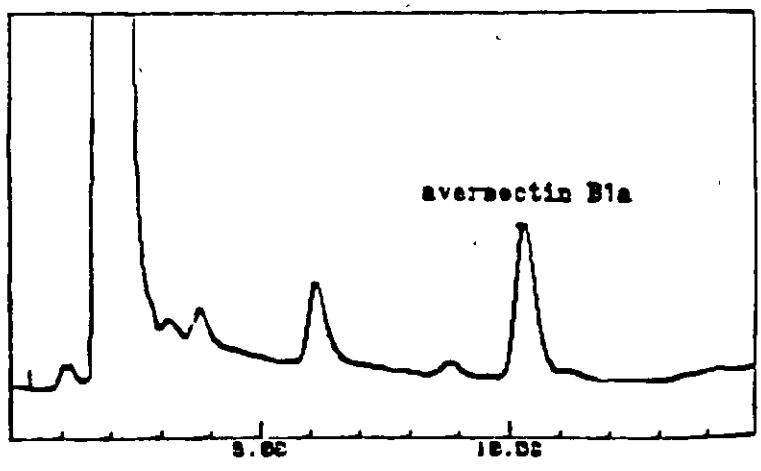
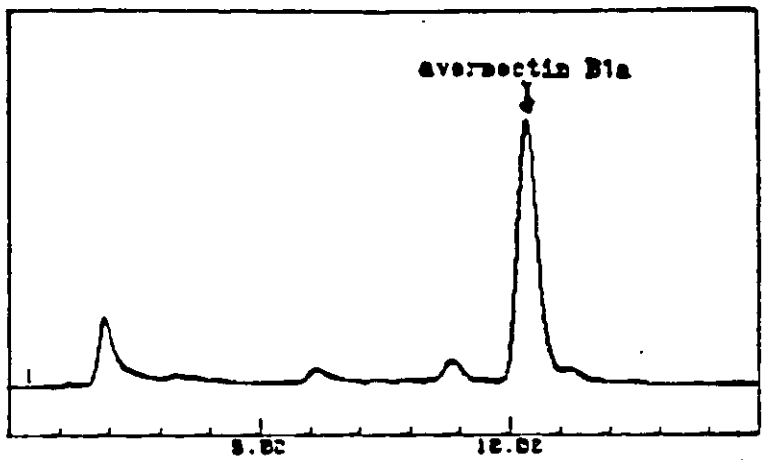
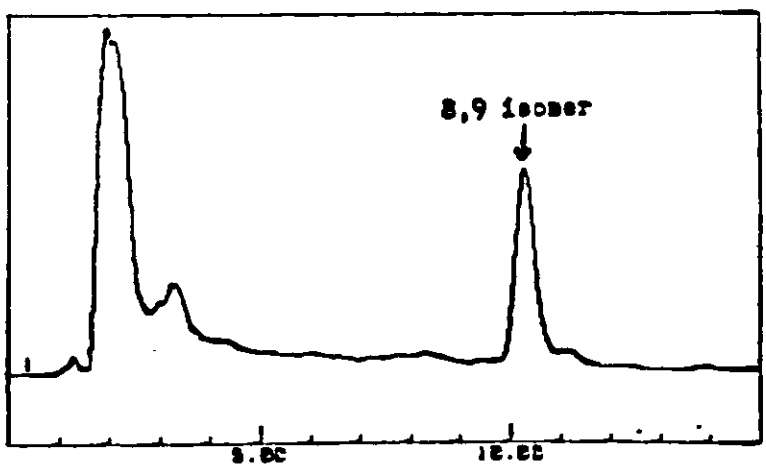


Figure 3. Typical Chromatograms of Avermectin B1 standard and Avermectin B1 8,9 Isomer in Cucumber .

8.5 NG/ML STD AVERMECTIN B1a



5.2NG/G 8,9 FORTIFIED CUCUMBER



III. PRINCIPLE

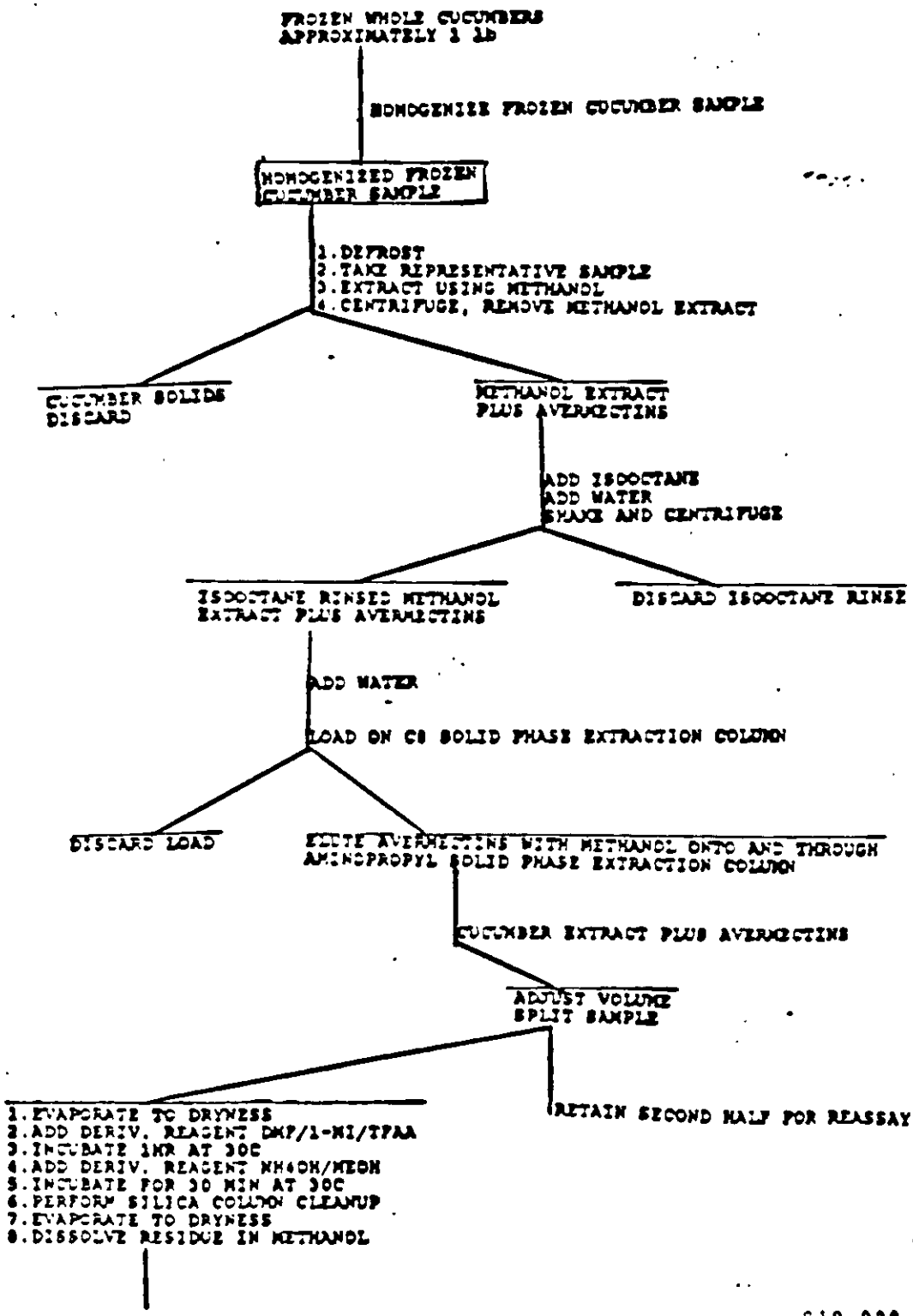
Ten gram samples of ground cucumbers are aliquoted into 50 ml disposable polypropylene centrifuge tubes with screw caps. The samples are extracted with methanol.

Residues of avermectin B1 and its 8,9 isomer are extracted from the cucumber homogenate by blending with methanol. The extract is centrifuged and made more aqueous by the addition of water. The aqueous solution washed with isooctane is passed through a C8 column, loading the avermectins onto the column. The eluant is discarded. The C8 column is attached to two preconditioned aminopropyl columns. The avermectins are eluted from the C8 column and through the aminopropyl columns with methanol. This eluant is diluted to 10 ml using methanol. The sample is split, evaporated to dryness and a fluorescent derivative is formed by reaction with a mixture of N,N-dimethylformamide, trifluoroacetic anhydride and 1-methylimidazole (Reagent A-1) for 1 hour at 30°C, followed by reaction with methanolic ammonium hydroxide (Reagent A-2) for 1/2 hour at 30°C. The reaction mixture is dissolved in chloroform and passed through a silica gel column for separation of the derivatized residue from derivatization reagents. The eluant is taken to dryness and dissolved in methanol. The derivatized residue is analyzed by reversed-phase liquid chromatography with fluorescence detection. As derivatization of the 8,9 isomer produces the same derivative as avermectin B1, the derivatized residue quantitated represents the sum of avermectin and its 8,9 isomer as shown below:

Parent Avermectin + 8,9 isomer —(heat, Reagent A)→ single fluorescent peak

A flow chart illustrating the analytical methodology used for this assay is given in Figure 4.

Figure 4. Analytical Methodology for the Analysis of Avermectins in Cucumbers



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NOTES TO THE ANALYST

Avermectin B1 has a very low water solubility, approximately 8 ppb. In addition, there are indications that this compound will form a monolayer at phase boundaries [liquid-air, liquid-liquid, or liquid-solid (glass or plastic) interface]. Because of these properties, loss of avermectin B1 due to adsorption to glassware may be critical when working in the low ppb range. Care should be taken to not take samples to dryness whenever possible. When samples are taken to dryness, sonication upon dissolution is crucial.

Unless otherwise specified, volumes described in this method should be measured by pipets and not approximated.

Suggested assay stopping points are after steps 7, 14, 15, and 28. These stopping points are for storage of samples overnight or over the weekend at 4°C or below.

IV. METHOD

A. Sample Preparation

1. The entire sample must be processed using a Hobart or Cuisinart food processor or equivalent. Grind the frozen sample until a homogeneous blend is attained.

2. Store an appropriate amount (usually approximately 500 grams of each treated sample and approximately 1000 grams of each control sample) in square 500 ml Nalgene bottles in a freezer until analysis.

NOTE: While processing the sample, dry ice may be added if the sample begins to thaw.

B. Extraction From Crop

3. Weigh out exactly 10 grams of the ground sample into a 50 ml disposable polypropylene centrifuge tube fitted with a screw cap.

4. Add 10 ml methanol. Position the Polytron probe near the bottom of the tube and homogenize on a setting of seven (25 for Polytron PT3000) for 30 seconds, or until homogeneous (but not less than 30 sec).

5. Rinse the probe with a 10 ml aliquot of methanol contained in a second 50 ml centrifuge tube by running briefly at a low speed. Further rinse the probe with methanol between samples to prevent cross contamination.

6. Centrifuge the initial homogenate for 5 minutes at 1500 RPM.

7. Pour the methanol solution into a clean 50 ml polypropylene centrifuge tube leaving the centrifuged cake behind.
Note: for matrices such as gerkin cucumbers where samples are fibrous, remove methanol with a disposable pipet to minimize transfer of fibrous pulp.

8. Transfer the 10 ml of methanol from the rinse tube into the tube with the cucumber cake. Rinse the tube with 2 ml methanol and add it to the cake tube. Vortex the sample tube and shake well for about 15 seconds to further extract the cake then centrifuge again for 5 minutes. Combine the methanol extracts.

C. Isooctane partition

9. Add 10 ml isooctane to the tube containing the methanol extract. Stopper, shake for approximately 10 seconds. Add 5 ml distilled water and shake again for approximately 30 seconds. Centrifuge for 5 minutes.

10. Remove and discard the upper isooctane layer. Repeat the extraction using a second 10 ml aliquot of isooctane and centrifuging for five minutes.

11. Transfer the purified methanol extract to a 100 ml graduated cylinder fitted with a ground glass stopper. Adjust the volume to 100 mL using distilled water.

Note: The solution should be at least 70% aqueous.

D. C-8/Aminopropyl Column Clean-up

12. Attach a 1000 mg/6 mL C8 solid phase extraction column fitted with an adapter and a 75 mL reservoir to the vacuum manifold. Condition the column by rinsing it with a 5 mL volume of methanol, followed by a 10 mL volume of Milli-Q water.

13. Transfer most of the aqueous methanol extract from the graduated cylinder to the 75 mL reservoir fitted with the C8 solid phase extraction column. Use the Spe-ed Mate-30 (or other manifold) and a 2 liter vacuum trap to load the sample. Maintain a vacuum of 10 in. of Hg and load the remainder of the sample. Dry the column by drawing a vacuum through it for

approximately 15 minutes. Using this setup, 12 to 15 samples can be loaded in parallel. Discard the eluant.

14. Remove the vacuum. Label the C8 columns with the sample number and remove them from the manifold. Attach 2 stacked 500 mg/6ml aminopropyl columns to the vacuum manifold and condition the columns with 2 column volumes of methanol. Place 15 mL silylated graduated centrifuge tubes in the manifold for collection of the samples. Attach the C-8 columns and reservoirs to the upper aminopropyl columns.

15. Rinse both the 50 mL centrifuge tube and the 100 mL graduated cylinder with 10 mL methanol. Pour the 10 mL of methanol from the graduated cylinder into the 75 mL column. Apply a vacuum (10 in. Hg or less) and collect the eluant in the silylated centrifuge tubes.

16. Adjust the volume in the centrifuge tube to exactly 10 mL with methanol using the graduations on the tube. Stopper and mix well by vortexing and shaking.

17. Transfer half (5 mL) of the sample to a suitable tube for storage using a disposable pipet. Store the retained half at -10C.

E. Derivatization

18. Take the half of the sample in the silylated centrifuge tube to dryness with nitrogen evaporation using heat lamps or a water bath at 50°C to 70°C.

NOTE: Care must be taken to insure that no moisture is present in the tubes prior to the addition of the derivatization reagent. This is particularly true for the samples since some water may be eluted from the column during step 15. Standards should be derivatized with unknowns (see section F.)

19. Add 0.2 mL of the trifluoroacylation derivatization reagent to the tube, stopper, vortex and sonicate to dissolve the residue.

20. Centrifuge briefly, and put unknowns and standards together in a 30°C water bath for one hour.

21. Remove the tubes and add 0.1 mL of the methanolic ammonium hydroxide reagent. Vortex and return stoppered tubes to the 30°C water bath for 30 minutes.

22. Remove the tubes from the bath, add 4 mL chloroform to each tube and vortex. The sample should appear white and cloudy after the addition of the chloroform.

23. Wash a silica cartridge (World Wide Monitoring or

equivalent) with 5 mL of chloroform using vacuum of approximately 10 in. of Hg to pull the chloroform from a reservoir into a waste collection tube.

24. After removal of the vacuum, place a 15 mL graduated centrifuge tube under the silica cartridge and add the sample in the 4 mL of chloroform to the cartridge using a Pasteur pipet. Apply the vacuum and collect the eluant in a 15 mL graduated centrifuge tube.

25. Remove the vacuum. Wash the silylated centrifuge tube two times with 2 mL of chloroform. Use the same pipet that was used to transfer the sample into the cartridge reservoir to transfer the chloroform wash into the reservoir. Apply the vacuum between washes only.

26. Elute the column with an additional 5 mL of chloroform to give a final eluant volume of approximately 13 mL.

27. Evaporate the chloroform with nitrogen in a 70°C water bath. Samples should be reduced to an oily residue, less than 0.1 mL.

28. Pipet exactly 5.0 mL methanol into the tube, vortex and sonicate to completely dissolve the residue.

NOTE: The amount of methanol used as the final volume may vary depending on the expected residue level. The volume should be measured precisely.

29. Centrifuge briefly prior to injection of the supernatant on HPLC.

F. Preparation of the Standards and Quantitation

Analytical Standards-

Avermectin B1 (L-676,863-038A002 or most recent reference standard lot.)
Avermectin B1a 8,9 isomer (L-652,280-002T001 or most recent reference standard lot.)

Chemical Data,
Merck Sharp & Dohme Research Laboratories,
P.O. Box 2000
Rahway, NJ 07065.

Preparation

1. To prepare 25 ug/mL stock solutions of avermectin B1a, and the B1a 8,9 isomer, weigh out enough of the standard glycerol formal solutions to produce 50 mL of a 25 ug/mL solution for avermectin B1a (ca.130 mg), and 25 mL of a 25 ug/mL solution for the 8,9 isomer of avermectin B1a (ca.165 mg). The standards should be prepared separately and diluted with acetonitrile. The above-cited reference standard avermectin B1 glycerol formal solution contains 0.956% avermectin B1a and 0.071% avermectin B1b w/w. The reference standard avermectin B1a 8,9 glycerol formal solution contains 0.38% w/w of the avermectin B1a 8,9 isomer. The exact concentration of all standards used should be reported and used throughout all calculations. The 25 ug/mL stock solution of avermectin B1a can be used to prepare a 50 ng/mL avermectin B1b standard solution.

2. To prepare 500 ng/mL intermediate stock solutions of the above standards, pipet 2 mL of the 25 ug/mL stock standard into separate 100 mL volumetric flasks. Make to volume with acetonitrile. Store standards in the dark at -20°C or lower. Label all glassware and standard storage containers with exact concentrations, notebook reference and the date of preparation. The 50 ng/mL standards can be prepared by diluting the 500 ng/mL intermediate stock solutions 1 to 10.

3. To prepare 2.0, 4.0, 6.0, 8.0 and 10.0 ng/mL working standards for the derivatization, transfer to separate silylated 15 mL tubes 0.2, 0.4, 0.6, 0.8 and 1.0 mL, respectively, of the 50 ng/mL avermectin B1a standard solution.

4. Reduce volume of standards to dryness. Derivatize and perform all subsequent operations for injection on the HPLC, as described above (steps 19-29).

V. REAGENTS

Solvents- acetone, chloroform, isooctane, methanol-EM Science or Burdick and Jackson (Pesticide Grade or Distilled in Glass); Sylon CT- Supelco Inc.; Ultrapure water-Milli-Q water purification system.

Solid phase extraction columns- Octyl (C-8) 1000 mg/6 mL, cat. no.606406, Analytichem International; Aminopropyl (NH_2) 500 mg/3 mL, cat no. 611303, Analytichem International; Silica 1000mg/6ml World Wide Monitoring, Horsham Pa.

NOTE: Recovery of avermectin B1 should be determined for each new lot of bonded phase columns.

HPLC Solvents- Prepare HPLC solvent by diluting 140 mL of Milli-Q purified water to 2.0 liters using HPLC grade methanol. Vacuum filter mixed HPLC solvent through Rainin 47 mm nylon 66 filter, 0.45 micron pore size. Equivalent filtering or degassing systems may be used.

Derivatization Reagents- Trifluoroacetic anhydride - Pierce; N,N-dimethylformamide - J. T. Baker; acetic anhydride, 1-methylimidazole - Aldrich; ammonium hydroxide (28-30%) -Fisher Scientific or Mallinckrodt.

To prepare the trifluoroacetyl derivatization reagent, add 0.4 mL 1-methylimidazole to 3.6 mL dimethylformamide in a 15 mL centrifuge tube, mix. Place the tube in an ice bath, allow the solution to chill for a minute, then slowly add 0.6 mL trifluoroacetic anhydride. Vortex until the solution is thoroughly mixed. Use this reagent immediately after preparation.

To prepare the methanolic ammonium hydroxide reagent, add 0.2 mL of ammonium hydroxide reagent (28-30% ammonia) to 3 mL methanol. Vortex to mix. This volume should be added using a 1 mL graduated pipet. Volumetric pipets should not be used for the preparation of this reagent because the high viscosity of the solution causes errors in the volume retained in the tip of the pipet.

These reagents must be prepared just prior to derivatization. This procedure provides enough of each reagent to derivatize 15 samples and 5 standards.

VI. GENERAL APPARATUS

Homogenizer- Brinkmann Instruments Polytron Blender model PT3000 with probe generator model PT-DA3012/2T or equivalent

Crop Processor- Hobart Food Processor or Cuisinart food processor model DLC-X.

Centrifuge- IEC Tabletop Model HN-S II or equivalent.

Sonicator- L&R Transistor/Ultrasonic model T-21 or equivalent.

Vacuum manifolds- Applied Separations Spe-ed Mate-30 or equivalent.

15 mL Graduated Centrifuge tubes- Fisher Scientific or equivalent.

To prepare silylated 15 mL graduated centrifuge tubes used for the derivatization reaction, silylate approximately once every two months using the following procedure: Fill each tube to the top with Sylon-CT. Let stand 20 minutes. Rinse thoroughly with toluene followed by methanol. Fill with methanol. Let stand 20 minutes, rinse thoroughly with acetone and dry. Following use, tubes should be cleaned by hand by first soaking in methylene chloride and then in detergent for at least several hours each, followed by thorough rinsing with hot water, distilled water, and acetone before reuse. As the derivatization reaction requires anhydrous conditions, tubes should be absolutely free of moisture prior to use. Oven drying ensures this condition.

VII. HPLC APPARATUS

Pump- LDC/Milton Roy ConstaMetric III Metering Pump

Injector-VICI Valco Model EQ-60

Pre-column- Whatman CSK 7cm x 2.1mm ID Pellicular ODS

Analytical column- ES Industries, Chromegabond MC18 column, 15 cm X 4.6 mm, 3 micron particle size, or equivalent.

Fluorescence Detector- SpectroVision FD100 Filter Fluorimeter equipped with a Xenon Flash light source, 365 nm excitation filter, 418 nm emission cutoff filter

Equivalent apparatus may be substituted.

VIII. HPLC OPERATING CONDITIONS

Mobile phase- 7% ultrapure water in methanol (v/v); flow

rate-1.0 mL/min; injection volume-50 microliters; detector parameters: Autozero= 50, Lamp frequency = 100, Response factor = 5, Range = 20, column temperature 28°C. (These conditions are for the ES Industries Chromegabond MC18 columns). Columns such as the Dupont Zorbax ODS may be substituted. Retention times for the fluorescent derivatives of avermectin B1a/8,9 isomer and avermectin B1b are approximately 10 minutes and 8 minutes, respectively, using the Chromegabond column and the above conditions. Using a column such as the Zorbax column, would result in different retention times for the two compounds.

Note: These conditions are for the HPLC equipment and columns described above. With other equipment or columns, minor changes in operating conditions may be required to obtain equivalent performance.

IX. DETERMINATION

Residues of avermectin B1a/8,9 isomer below 2 ng/g are non-detectable (reported as ND). The peaks representing avermectin B1a/8,9 residues between 2-5 ng/g are identified but not quantitated (reported as NQ) and the peaks for residues above 5 ng/g are identified and quantitated. Since avermectin B1b is at most 20% (usually less than 10%) of the active ingredient, residue levels are generally less than the quantitation limit (5 ng/g) or the detection limit (2 ng/g). The peak representing avermectin B1b is identified but not quantitated when the residue level is between 2-5 ng/g. Residues of avermectin B1b above 5 ng/g are identified and quantitated in the same manner as the avermectin B1a/8,9 isomer, using the avermectin B1a standard curve for quantitation.

An analysis set is comprised of no less than 5 standards and no more than 15 samples, including recovery or control samples. The standards are run before and after the samples to ensure the stability of the HPLC system, the standards and the samples. For each analysis set, the slope and intercept are determined from the linear regression curve fit of the standards' peak height vs. concentration in nanograms per milliliter. Occasionally it has been observed that the peak height for both injections of a single standard is much lower than expected. Because it is known that this observation can be attributed to low derivatization reaction yield, the data for this standard may be discarded in determining the regression coefficients.

The concentration of avermectin B1a/8,9 isomer in a residue sample is determined as follows:

$$C=(PK\ HT-I)/S \qquad UNK=(CxFV)/(SW \times FRAC)$$

Where:

C=concentration of avermectin B1a/avermectin 8,9 isomer in ng/mL in the final volume used for HPLC analysis, PK HT= peak height of avermectin B1a/8,9 isomer derivative, I= intercept, S= slope, FV= final volume used for HPLC analysis, SW= sample weight, UNK= concentration of avermectin B1a/8,9 isomer in ng/g in the unknown residue sample. Frac= Fraction of the sample used for the assay (usually 0.5). Avermectin B1b residues are calculated in the same manner.

X. BIBLIOGRAPHY

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